Atherosclerosis is a progressive disease characterized by lipid accumulation, inflammation, cell death, and fibrosis within the artery wall. From the initial phases to eventual rupture of the vulnerable atherosclerotic plaque, inflammatory mediators play a key pathophysiological role. Pathological studies revealed that the occurrence of acute plaque rupture and atherothrombotic clinical events (i.e., myocardial infarction or ischemic stroke) depends on the plaque composition rather than the degree of stenosis. Vulnerable plaques are characterized by thin fibrous caps, a large lipid core, and increased inflammation. The thickness of fibrous caps depends on the balance between extracellular matrix production through smooth muscle cells (SMCs) and degradation, because of matrix metalloproteinases (MMPs) secreted by inflammatory cells. MMPs have been described to destabilize vulnerable plaques by promoting angiogenesis, leukocyte infiltration, and apoptosis. Moreover, an unexpected role for neutrophils in the pathogenesis of atherosclerotic plaque vulnerability has been recently identified.

Recent experimental and clinical data suggest that local and systemic elevation of endocannabinoid levels are linked with the development of atherosclerotic vascular disease and coronary circulatory dysfunction in obese individuals, a precursor of coronary artery disease. However, it remains unclear whether endocannabinoid levels might represent a risk factor or diagnostic biomarker for acute atherosclerotic vascular events. In fact, a causal role of increased endocannabinoid levels in atherosclerotic plaque vulnerability and occurrence of acute clinical events has not been investigated.

Endocannabinoids are lipids and belong to a physiological system that is further composed of the 2 known receptors, CB₁ and CB₂, and enzymes for endocannabinoid biosynthesis and inactivation. A major limitation for in vivo studies of the...
role of endocannabinoids in (patho)physiological conditions is their rapid metabolism, which led to the development of mice lacking the major anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), for studying the physiological functions of anandamide. These mice have strongly increased anandamide levels, in particular, in brain and liver, confirming the key role of FAAH in anandamide degradation.20,21

In this study, we interbred FAAH−/− mice with apolipoprotein E–deficient (ApoE−/−) mice to assess the impact of FAAH deficiency on atherosclerotic plaque vulnerability.

Materials and Methods

For expanded methods, see the online-only Data Supplemental materials.

Animals and Atherogenic Diet

ApoE−/−FAAH−/− mice were in the C57BL/6J background20 and crossed with ApoE−/− mice to generate ApoE−/−FAAH−/− mice. After backcrossing for at least 8 generations, 10-week-old ApoE−/− and ApoE−/−FAAH−/− mice were fed with high-cholesterol diet (HCD): 1.25% cholesterol, 0% cholate; Research Diets; D12108) and kept in conventional housing. All animal studies have been approved by the Swiss Federal Veterinarian Office and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Blood Analysis

For measurements of differential blood cell counts (hematocytometer; Sysmex Digitana AG), serum cholesterol (Infinity, Thermo Electron Corporation), and triglyceride content (Cayman Chemical), blood samples were collected after overnight fasting before and after 5, 10, and 15 weeks of HCD.

Endocannabinoid Measurement

Endocannabinoids were extracted from 100 µL of mouse serum or 20 mg of heart tissue, and analyses were performed on a liquid chromatography-tandem mass spectrometry system, as described.22

Histological and Morphometric Analysis

Atherosclerotic lesions were analyzed within the thoracoabdominal aorta and aortic sinus by Sudan IV staining for lipid deposition. Immunostaining was performed with the following antibodies: monoclonal CD68 (AbD serotec), CD3 (Pharmingen), Ly6G (Pharmingen), or polyclonal smooth muscle myosin (Biomedical Technologies), MMP-8, MMP-9 (both R&D Systems), and CXC ligand 1 (CXCL1) (KC/GROx, Santa Cruz Biotechnology). Apoptotic neutrophils were stained with the DeadEnd fluorometric TUNEL System (Promega), and counterstained with Ly6G and Alexa Fluor 594-labeled secondary antibody. Collagen was stained with 0.1% Sirius Red (Sigma Aldrich), and arterial smooth muscle staining with the DeadEnd fluorometric TUNEL System (Promega), and counterstained with Ly6G and Alexa Fluor 594-labeled secondary antibody. Collagen was stained with 0.1% Sirius Red (Sigma Aldrich), and total collagen content as well as type I and III fibers were evaluated under polychromatic light or polarized light illumination, respectively.

Intravital Microscopy

Intravital microscopy of the carotid artery was performed in ApoE−/−Cx,cr1cre+ mice, as described.11 Therefore, mice were fed with HCD for 10 weeks and randomly assigned to receive FAAH inhibitor URB597 (1 mg/kg intraperitoneally; Cayman Chemical) or vehicle (2% dimethyl sulfoxide, 2% Tween-80 in saline) during the last 2 weeks of HCD. A phycoerythrine-conjugated antibody to Ly6G (clone IA8, 1 µg) was instilled via a jugular vein catheter, 15 minutes before recording.

Flow Cytometry and ELISA

Blood was drawn by cardiac puncture, and bone marrow was obtained by flushing femurs. Both were subjected to red blood cell lysis. Harvested and dissected aortic arches were enzymatically digested.23 Staining for flow cytometry was performed with the following antibodies: CD4-PerCP (RM4-5), CD25-PE (PC61), CD45-FITC (104), Ly6G-PE (1A8), and CD11b-APC (M1/70; all from BD Biosciences). Foxp3-FTTC (FJK-16s) antibody was from eBioscience. Dead cells were excluded from analysis by labeling with 7-amino-actinomycin D (Invitrogen). All flow cytometry samples were collected and analyzed with the Accuri C6 flow cytometer and CFlow Plus Software (BD Biosciences). Granulocyte colony-stimulating factor in mouse serum was measured by ELISA (R&D Systems).

Splenocyte Stimulation for Cytokine Measurement

Murine splenocytes were isolated by homogenization and dissociation through 70-µm cell strainers, followed by red blood cell lysis. Cells (2×10^6/mL) were stimulated in Roswell Park Memorial Institute 1640 medium +10% fetal calf serum with plate-bound CD3 (10 µg/mL; 145-2C11; BD biosciences), and cell culture supernatants were harvested after 48 hours for quantification of interferon-γ, tumor necrosis factor-α (TNF-α), interleukin (IL)-10, and IL-17 by ELISA (R&D Systems).

Real-Time PCR

Messenger RNA expression of inflammatory markers (for extended description and primers see Table I in the online-only Data Supplement) was normalized to hypoxanthine-guanine phosphoribosyltransferase, and the fold induction was calculated by the comparative Ct method.

Statistical Analysis

All results are expressed as mean ± SEM. Differences between probability values <0.05 were considered significant. Unpaired 2-tailed t test or 1-way ANOVA with Bonferroni’s posttest was used.

Results

FAAH Deficiency Leads to Systemic Elevation of Its Substrates

In addition to anandamide degradation, FAAH is also partly responsible for the regulation of the levels of other N-acylethanolamines, which do not bind to cannabinoid receptors, but nevertheless can exert antiinflammatory or lipolytic effects via peroxisome proliferator-activated receptor-α, that is, PEA, and OEA, respectively.24–26 Serum levels of anandamide, PEA and OEA levels were significantly higher in young ApoE−/−FAAH−/− mice compared with ApoE−/− controls (Figure 1A–1C). Their levels even increased in ApoE−/−FAAH−/− under HCD, peaking after 5 (PEA) or 10 weeks of HCD (Figure 1D). Comparable differences in FAAH substrate levels were found in hearts of ApoE−/−FAAH−/− mice with maximum levels after 10 weeks of HCD (Figure 1D). Comparative differences in FAAH substrate levels were found in hearts of ApoE−/−FAAH−/− and ApoE−/− mice (Table II in the online-only Data Supplement).

Remarkably, absence of FAAH did affect total cholesterol levels in young mice (371.1±20.2 mg/dL for ApoE−/− versus 218.3±23.2 mg/dL for ApoE−/−FAAH−/−; P<0.001), whereas no difference was observed after HCD feeding (Table III in the online-only Data Supplement). We also analyzed triglyceride levels after 10 weeks of HCD, which were comparable. However, we found a significant difference in body weight between both genotypes in young mice (22.0±0.4 g for ApoE−/− versus 24.8±0.3 g for ApoE−/−FAAH−/−; P<0.001) that was again no longer found after HCD.
FAAH Deficiency Leads to the Development of Smaller Atherosclerotic Plaques

Within each genotype, plaque sizes significantly increased with duration of HCD. However, the comparison between both genotypes revealed that plaque areas in descending thoracoabdominal aortas were substantially ($\approx$50%; $P=0.007$) smaller in ApoE$^{-/-}$FAAH$^{-/-}$ mice after 15 weeks of HCD (Figure 2A). Similarly, we found smaller plaque areas in aortic sinuses of ApoE$^{-/-}$FAAH$^{-/-}$ mice compared with ApoE$^{-/-}$ mice after 10 to 15 weeks of HCD ($P=0.01$ or 0.027, respectively; Figure 2B).

Plaques in FAAH-Deficient Mice Have a More Vulnerable Phenotype

The cellular composition of atherosclerotic lesions rather than plaque size appears crucial in both atherogenesis and plaque rupture. We therefore assessed the effect of FAAH deficiency on atherosclerotic lesion quality by immunohistochemical analysis of aortic sinus cross sections. Although the content of macrophages was comparable between the 2 genotypes at all time points (Figure 3A), the number of T-lymphocytes was significantly (1.7-fold; $P=0.019$) reduced after 10 weeks in ApoE$^{-/-}$FAAH$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (Figure 3B). At the same time point, we found a significantly higher neutrophil content ($P=0.02$) in plaques of ApoE$^{-/-}$FAAH$^{-/-}$ compared with ApoE$^{-/-}$ mice (Figure 3C). High neutrophil infiltration is strongly associated with features of vulnerable plaques.12,14,15 Conversely, the content of SMCs, which reflects plaque stability, was reduced by 36% after 10 weeks of HCD ($P=0.01$; Figure 3D).

We further assessed the expression of proteases, which are released by infiltrating inflammatory cells within plaques of ApoE$^{-/-}$ and ApoE$^{-/-}$FAAH$^{-/-}$ mice. The expression of MMP-9, an enzyme involved in the degradation of plaque-stabilizing matrix protein, was increased by 73% ($P=0.049$) in ApoE$^{-/-}$FAAH$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (Figure 3E). MMP-8 expression was weak and not affected by FAAH deficiency (Figure 3F). Despite the increase in MMP-9 expression, the total plaque collagen content was unchanged ($P=0.9$; Figure 3G). In addition, the plaque composition of collagen type I and type III fibers was comparable between the genotypes.

MMP-9 Expression Colocalizes With Plaque Neutrophils

To confirm the role of neutrophils in plaque vulnerability, we verified the cellular source of plaque MMP-9 expression by double immunofluorescence staining. In both ApoE$^{-/-}$ and ApoE$^{-/-}$FAAH$^{-/-}$ mice, MMP-9 mainly colocalized with neutrophils rather than macrophages, and the staining mostly appeared intracellularly (Figure I in the online-only Data Supplement).

Increased Proinflammatory Cytokine Expression, but No Increase of Ly6C$^{hi}$ Monocytes in FAAH-Deficient Mice

Despite the predominant evidence for increased plaque vulnerability in ApoE$^{-/-}$FAAH$^{-/-}$ mice, the reduced T-lymphocyte counts revealed by immunohistological analysis (Figure 3B) are puzzling. A potential explanation for the difference in plaque T-lymphocyte numbers might be a modulation of T-lymphocyte subtype commitment in FAAH-deficient mice. After 10 weeks of HCD, we found a significantly reduced number of CD4$^+$ effector T-cells and FoxP3$^+$ regulatory T-cells in spleens of FAAH-deficient mice (Figure 4A). Concomitantly, in vitro stimulation of splenocytes with plate-bound CD3 revealed a significantly increased production of Th1 cytokines interferon-$\gamma$ and TNF-$\alpha$, whereas IL-10

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**Figure 1.** Circulating levels of fatty acid amide hydrolase (FAAH) substrates anandamide (AEA; A), palmitoylethanolamide (PEA; B), and oleoylethanolamide (OEA; C), as well as the non-FAAH substrate 2-arachidonoylglycerol (2-AG) (D) in apo-lipoprotein E-deficient (ApoE$^{-/-}$) (white bars; $n=8$–10) compared with ApoE$^{-/-}$FAAH$^{-/-}$ mice (black bars; $n=6$–10). *$P<0.05$; **$P<0.01$; ***$P<0.001$ for 2 group comparison between genotypes at same time point. $P<0.05$; **$P<0.01$; ***$P<0.001$ for multiple group comparison between 0 and 5, 10, or 15 weeks high-cholesterol diet-fed mice within each genotype.
compared with ApoE–/– mice (Figure 5A), confirming our prediction that enhanced recruitment of neutrophils might account for their increased numbers in aortic root plaques and arches. To support our hypothesis, we performed intravital microscopy in HCD-fed ApoE–/–Cx3cr1egfp/+ mice carrying fluorescent monocytes. To visualize neutrophils, an antibody to Ly6G was instilled intravenously (Figure 5D). Treatment of mice with the selective FAAH inhibitor URB597 significantly increased the number of adherent neutrophils along the carotid artery (Figure 5E), whereas the number of adherent monocytes was comparable between URB597- and vehicle-treated mice (Figure 5F).

FAAH Deficiency Does Not Affect Neutrophil Secretion and Migration In Vitro

The enhanced neutrophil recruitment in plaques of ApoE–/–FAAH–/– mice might be a consequence of altered cellular responses to inflammatory activation. To test this hypothesis, we performed in vitro experiments with freshly isolated neutrophils from ApoE–/–FAAH–/– and ApoE–/– mice. No difference in chemotactic migration was found, and chemokine-induced MMP-9 release and proteolytic activity was comparable between the 2 genotypes (Figures III–V in the online-only Data Supplement).

Local Increase of the Neutrophil Chemoattractant CXCL1 in FAAH-Deficient Mice

We further determined transcript levels of neutrophil chemoattractants in abdominal aortas after 10 weeks of HCD by real-time PCR. We found a 1.9-fold increased mRNA expression of CXCL1 (P=0.004), and a nonsignificant 1.5-fold increase of CC ligand 3 (P=0.1) in ApoE–/–FAAH–/– mice (Figure 6A). TNF-α and CXCL2 levels were not affected by FAAH deficiency. Similarly, mRNA levels of chemokine receptors CXCR2 and CXCR4, as well as E-selectin and P-selectin were not significantly different (Figure 6B). Systemic chemokine levels were comparable between the 2 genotypes after 10 weeks diet (Table IV in the online-only Data Supplement), which supports the hypothesis of locally increased CXCL1 chemotactant release that favors neutrophil infiltration in plaques of ApoE–/–FAAH–/– mice. We confirmed the local CXCL1 chemokine expression by immunohistochemistry, which appeared more pronounced in plaques of ApoE–/–FAAH–/– rather than ApoE–/– mice (Figure 6C).

Discussion

Emerging evidence suggests an activation of the endocannabinoid system in various pathophysiological conditions, including atherosclerosis. Increased systemic endocannabinoid anandamide and 2-arachidonoylglycerol levels have been found in coronary artery disease patients, as well as increased CB1

Increased Neutrophil Counts in Aortas, but Not Peripheral Blood and Bone Marrow

Hyperlipidemia has been shown to induce neutrophilia in mice because of enhanced granulopoiesis and mobilization from the bone marrow. To verify the hypothesis that the increased neutrophil plaque content might be a consequence of systemically increased neutrophil counts, we assessed neutrophil counts in peripheral blood, bone marrow, and enzymatically digested aortic arches. However, the flow cytometric analysis did not reveal a difference in circulating and bone marrow CD45+CD11b+Ly6G+ neutrophil counts between the 2 genotypes. Conversely, the number of neutrophils was significantly increased within aortic arches of ApoE–/–FAAH–/– compared with ApoE–/– mice (Figure 5A), confirming our immunohistological findings (Figure 3C). No difference in granulocyte colony-stimulating factor serum levels was found (Figure 5B), suggesting that FAAH deficiency does not affect granulopoiesis.

Another possible explanation for the increased neutrophil plaque content in ApoE–/–FAAH–/– mice could be reduced cell death. However, we did not find a difference in the number of TUNEL+ apoptotic neutrophils within the plaques (Figure 5C), suggesting that rather enhanced recruitment of neutrophils might account for their increased numbers in aortic root plaques and arches. To support our hypothesis, we performed intravital microscopy in HCD-fed ApoE–/–C57Bl/6J mice carrying fluorescent monocytes. To visualize neutrophils, an antibody to Ly6G was instilled intravenously (Figure 5D). Treatment of mice with the selective FAAH inhibitor URB597 significantly increased the number of adherent neutrophils along the carotid artery (Figure 5E), whereas the number of adherent monocytes was comparable between URB597- and vehicle-treated mice (Figure 5F).

Figure 2. Lesion size and lipid staining in apolipoprotein E–deficient (ApoE–/–) white bars; n=10) and ApoE–/– fatty acid amide hydrolase-deficient (FAAH–/–) mice (black bars; n=6–9): A, Thoracoadominal aortas and B aortic sinuses. *P<0.05; **P<0.01 for 2 group comparison between genotypes at same time point. *P<0.05; **P<0.01; ***P<0.001 for multiple group comparison between 5 and 10 or 15 weeks high-cholesterol diet-fed mice within each genotype.
immunostaining in lipid-rich plaques, as compared with fibrous plaques.\textsuperscript{17} Moreover, we reported presence of endocannabinoids and their receptors (mainly CB\textsubscript{2}) in human carotid plaque specimens.\textsuperscript{28} In agreement with the published human data,\textsuperscript{17} we previously demonstrated a modulation of endocannabinoid levels during atherosclerosis development in mice.\textsuperscript{16} This may have functional relevance, given that CB\textsubscript{1} antagonism with rimonabant reduced atherosclerosis in low-density lipoprotein receptor-deficient mice.\textsuperscript{29} However, it is difficult to dissect the cardiometabolic effects of this drug from its antiatherosclerotic effects, and it was shown to mediate, in part, antiinflammatory effects independent of CB\textsubscript{1} receptor blockade.\textsuperscript{29} Thus, clear evidence for a causal role of enhanced endocannabinoid tone in atherosclerosis is missing.
This prompted us to clarify the involvement of enhanced FAAH substrate levels by genetic deficiency in the ApoE–/– mouse model of atherosclerosis. We used knockout mice with genetic ablation of the anandamide-metabolizing enzyme FAAH that have elevated endocannabinoid anandamide levels in brain and peripheral tissues. Conversely, in a model of doxorubicin-induced cardiotoxicity FAAH–/– mice developed increased myocardial injury. A limitation of the FAAH–/– model is certainly that FAAH has the capacity to metabolize a range of substrates, including PEA and OEA. Nevertheless, to the best of our knowledge, there is no loss-of-function mouse model with impaired anandamide synthesis available. In fact, at the level of anandamide (and related N-acylethanolamines) synthesis, alternative pathways appear to exist, given that genetic deficiency of enzymes involved in endocannabinoid synthesis does not impair N-acylethanolamines synthesis.

The elevated systemic and cardiac levels of FAAH metabolites in young ApoE–/–FAAH–/– mice compared with ApoE–/– mice confirm the major role of FAAH in the metabolism of anandamide, PEA, and OEA. The further increase of their serum levels under HCD feeding suggests that additional mechanisms, such as increased synthesis, may account for this observation. However, the finding that an increase of endocannabinoid levels in ApoE–/–FAAH–/– mice developing atherosclerosis. Importantly, we previously demonstrated that the increase of endocannabinoid levels in HCD-fed ApoE–/– mice was paralleled with atherosclerotic plaque progression and was not solely a diet-mediated effect.

The observed differences in weight and serum cholesterol levels at young age might be explained by the well-described effects of endocannabinoids on food intake and lipid levels in experimental and clinical studies. It is likely that these metabolic differences in young mice do not have a major impact on atherosclerosis development, as these differences were no longer found after 5 to 15 weeks because HCD feeding. Moreover, it was previously shown by Dol-Gleizes et al. that low-density lipoprotein receptor–deficient mice with reduced weight gain because of food restriction develop atherosclerosis comparable with ad libitum fed mice.

ApoE–/–FAAH–/– mice developed smaller plaques with an unstable phenotype, which is evidenced by the increased number of neutrophils and enhanced MMP-9 production, together with lower contents of SMCs. Conversely, the plaque macrophage content and Ly6Chi/lo ratio was unaffected. Surprisingly, the ratio of plaque collagen type I to type III fibers was unchanged. We even found a nonsignificant tendency for increased total collagen content in ApoE–/–FAAH–/– mice (P>0.09), which might be related to a profibrotic effect of endocannabinoids. This is supported by previous findings in experimental models of liver fibrosis and diabetic cardiomyopathy. Although MMP-9 is involved in extracellular matrix degradation, Sluijter et al suggested that MMP-9 could not be solely responsible for plaque vulnerability, as intact collagens are not substrates for MMP-9. They found that both MMP-8 and MMP-9 are associated with vulnerability of human carotid plaques. We may also speculate that MMP-9 is mostly stored in neutrophil granules rather than secreted to the extracellular space, which is supported by the double immunofluorescence analysis of MMP-9 and neutrophil marker Ly6G.

Neutrophils have been detected at the sites of plaque erosion or rupture on atherectomy specimens from patients with unstable angina, and on autopsy samples from patients with
Acute myocardial infarction. Accordingly, high neutrophil numbers have been recently associated with features of rupture-prone carotid plaques, that is, large lipid core, high macrophage numbers, and low collagen amount and SMC numbers. Presence of neutrophils in murine atherosclerotic plaques has been confirmed in various experimental studies including our own. Among other possible proatherogenic mechanisms, neutrophils have been recently shown to deposit cathelicidins (in mice known as cathelin-related antimicrobial peptide, [CRAMP]) on inflamed arterial endothelium, thereby promoting the recruitment of inflammatory monocytes.

Increased numbers of plaque neutrophil infiltrates have been attributed to hyperlipidemia-induced granulopoiesis and mobilization from the bone marrow. However, our flow cytometry and immunohistochemistry data suggest that increased neutrophil plaque contents in FAAH knockouts are rather a consequence of increased recruitment to atherosclerotic plaques than to enhanced granulopoiesis. In fact, circulating and bone marrow neutrophil numbers were comparable after 10 weeks of HCD in both genotypes, and no difference in serum levels of the granulopoiesis-stimulating factor granulocyte colony-stimulating factor was found. We excluded the possibility that the increased plaque neutrophil content was a consequence of altered apoptotic cell death, and we did not find a difference in neutrophil migration and MMP-9 secretion in response to inflammatory activation in vitro. However, the in vitro stimulation response was moderate in our experiments, possibly because of a preactivation state of...
neutrophils induced by the peritoneal lavage isolation. In the same context, we cannot exclude the possibility of increased intracellular MMP-9 storage or enhanced synthesis of other neutrophil secretory proteins, such as CRAMP, in FAAH-deficient neutrophils.

Nevertheless, the in vivo imaging confirmed that impaired FAAH activity results in specific recruitment of neutrophils, but not monocytes, to large arteries, suggesting a neutrophil-specific vascular activation pattern. As a possible mechanistic explanation, we found that the neutrophil chemokine CXCL1 was upregulated in aortas of ApoE−/−FAAH−/− mice, suggesting that increased local synthesis of CXCL1 contributes to the enhanced influx of neutrophils in plaques of ApoE−/−FAAH−/− mice. This is possibly linked to the proinflammatory Th1 cytokine pattern, as a result of reduced numbers of regulatory T-cells in FAAH-deficient mice.

A recent in vitro study reported that IL-17 and TNF-α synergistically mediate sustained neutrophil recruitment to activated endothelium, via specific induction of neutrophil chemoattractants (including CXCL1) and adhesion molecules.41 Thus, the 2.6-fold higher TNF-α production and 1.6-fold (nonsignificant; P=0.12) induction of IL-17 in FAAH-deficient mice might increase CXCL1 expression within atherosclerotic plaques. A limitation of our study is that it remains unclear whether the smaller plaque size in ApoE−/−FAAH−/− mice is a consequence of reduced SMC content as well as the high amounts of neutrophil proteases within the plaque, which is likely to induce extracellular matrix degradation. Although there was no difference in plaque collagen content, the proteolytic activity could affect other extracellular matrix elements, such as elastin and proteoglycans. The reduced SMC content might be a consequence of reduced migration and proliferation or enhanced necrosis/apoptosis, which deserves further investigation.

In conclusion, our data suggest that enhanced levels of FAAH substrates (such as anandamide) trigger the development of small plaques with high neutrophil and reduced SMC content, and thus increased risk of rupture. Thus, we may speculate that increased endocannabinoid anandamide levels found in patients with coronary artery disease might increase the risk for developing an acute clinical event because of plaque rupture.

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Disclosures
None.

References


Fatty Acid Amide Hydrolase Deficiency Enhances Intraplaque Neutrophil Recruitment in Atherosclerotic Mice

Sébastien Lenglet, Aurélien Thomas, Oliver Soehnlein, Fabrizio Montecucco, Fabienne Burger, Graziano Pelli, Katia Galan, Benjamin Cravatt, Christian Staub and Sabine Steffens

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Supplemental Material

Methods

Endocannabinoid measurement

Endocannabinoids were extracted from 100 µl of mouse serum by liquid-liquid extraction. In the same manner, endocannabinoids were extracted from 20 mg of heart tissue using a Mill MM400 homogenizer (Retsch, Germany). Analyses were performed on a LC-MS/MS system consisting of an Ultimate 3000 RS (Dionex, CA, US) LC system and a 5500 QTrap triple quadrupole/linear ion trap (QqQ LIT) mass spectrometer equipped with a TurboIon-Spray interface (AB Sciex, ON, Canada). Data acquisition and analysis were performed using Analyst software version 1.5.1 (AB Sciex, ON, Canada).¹

Histological and morphometric analysis

Mice were euthanized after 5, 10 or 15 weeks HCD. Vessels were rinsed with normal saline, aortic sinuses embedded in OCT compound (Tissue-Tek, Sakura, Netherlands) and snap-frozen. Thoraco-abdominal aortas were fixed in 4% PFA and opened longitudinally. Atherosclerotic lesions within the thoraco-abdominal aorta and aortic sinus were visualized by Sudan IV staining for lipid deposition. We calculated for each aortic sinus an average of lipid deposition from 6 sections (5 µm) separated by 50 µm from each other. Stained areas were calculated as percentage of the total vessel surface or cross-sectional area, respectively (MetaMorph software; Zeiss).

Immunostaining was performed on at least 3 acetone-fixed 5 µm cross-sections of aortic sinuses, using monoclonal antibodies for mouse macrophages (CD68; FA-11; AbD serotec), T lymphocytes (CD3; 17A2; Pharmingen), neutrophils (Ly6G; clone 1A8; Pharmingen), or polyclonal SMC marker smooth muscle myosin (SMM,
Biomedical Technologies), MMP-8, MMP-9 (both R&D Systems) and CXCL1 (KC/GROα, Santa Cruz Biotechnology). Binding of primary antibodies was detected with alkaline phosphatase system (Vector Laboratories). Stained areas were calculated as percentage of stained area per total lesional area or counted cells per lesional area in case of CD3.

Collagen was stained by incubation with 0.1% Sirius Red (Sigma) in saturated picric acid for 90 min. Sections were rinsed twice with 0.01 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 seconds and cover-slipping, the sections were analyzed by polychromatic or polarized light microscopy. Total collagen content was evaluated under polychromatic light. Interstitial collagen subtypes were evaluated using polarized light illumination; under this condition thicker type I collagen fibers appeared orange or red, whereas thinner type III collagen fibers were yellow or green.

**Double immunofluorescence staining**

Colocalization of macrophage (CD68) and neutrophil (Ly6G) immunosignals with MMP-9 was analyzed by double immunofluorescence staining with Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies (Jackson ImmunoResearch) and DAPI counterstaining of nuclei (Vector Laboratories).

**TUNEL staining**

Nuclear DNA fragmentation in situ was detected using terminal deoxynucleotidyl transferase incorporation of biotinylated deoxyuridine at the sites of DNA breaks in apoptotic cells. Three sections (5 µm) of mouse aortic roots separated by 50 µm from each other were stained with a fluorometric TUNEL assay kit according to the
manufacturer’s guidelines (Promega) and counterstaining with Ly6G and Alexa Fluor 594-labeled secondary antibody. Double-positive cells per aortic sinus cross-section were counted.

**Intravital microscopy**

Intravital microscopy of the carotid artery was performed in ApoE<sup>−/−</sup>Cx<sub>3</sub>cr1<sup>egfp/+</sup> mice as described. Therefore, mice were fed with HCD for 10 weeks and randomly assigned to receive FAAH inhibitor URB597 (1 mg/kg intraperitoneally; Cayman Chemical) or vehicle (2% DMSO, 2% Tween-80 in saline) during the last two weeks of HCD. A PE-conjugated antibody to Ly6G (clone 1A8, 1 µg) was instilled via a jugular vein catheter 15 min prior to recording. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a beam splitter to enable synchronized dual-channel recording, a Hamamatsu 9100-02 EMCCD camera, and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell software was used.

**Flow cytometry of Ly6C<sup>hi</sup> monocytes**

Whole blood and digested aortic arches were stained as previously described with following antibodies: Ly6C-FITC (AL-21), CD11b-APC (M1/70), CD90.2-PE (53-2.1), B220-PE (RA3-6B2), CD49-PE (DX5), NK1.1-PE (PK136), and Ly6G-PE (1A8; all from Pharmingen). F4/80-biotin (CI:A3-1; AbD Serotec), I-Ab-biotin (25-9-17), CD11c-biotin (both from BioLegend) and streptavidin-PerCP (Pharmingen) were used to determine macrophage and dendritic cell differentiation.
Real-time polymerase chain reaction (PCR)

Total RNA from mouse spleens and thoraco-abdominal aortas was extracted with TRIzol Reagent (Invitrogen). Reverse transcription was performed using the PrimeScript™ RT reagent kit (TaKaRa), and real-time PCR performed with the ABI Prism StepOnePlus Sequence Detection System (Applied Biosystems) using the ABsolute™ QPCR Mix (ABgene). Messenger RNA expression of inflammatory markers (Supplemental Table I) was normalized to HPRT, and the fold induction was calculated by the comparative C_t method.
Supplemental Table I. Primers and probes used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Primer sequence</th>
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| Cxcl1 (NM_008176)      | Fw  CATAGCCCACTCAAGAATGGT  
                        | Rv  TGAACCAAGGGAGCTTCAG  
                        | Probe  CGCGAGGCTTGCCTTGACC |
| Cxcl2 (NM_009140)      | Fw  AGTGAACTGCGCTGTAATG  
                        | Rv  GCCCTTGAGAGTGGCTATGA  
                        | Probe  AAGACCCTGCCAAGGGTTGACTTC |
| Cxcr2 (NM_009909)      | Fw  TCACAAACAGCGCTGTAATG  
                        | Rv  GCAAGAACGTCTACTAAATGTGAA  
                        | Probe  TGCAGGATTAAGTTACCTCAAAGATGGG |
| Cxcr4 (NM_009911)      | Fw  AGGGGCTCTAGACGAGATGTT  
                        | Rv  AGGGTCCCTTGAGAGTCA  
                        | Probe  CCATGGAACCGACGTGAGATGATAT |
| Ccl3 (NM_011337)       | Fw  CAGCCAGGTGTCATTTTCT  
                        | Rv  CCAAGACTCTCAGGGATTTCAG  
                        | Probe  AGGAGAAACCGGCAGATCTCGCT |
| Hprt (NM_013556)       | Fw  GACCGGTCCCGCTCATGC  
                        | Rv  TCATAACCTGGTTTACTACGTC  
                        | Probe  ACCCGCAGTCAGAGCTGTCGT |
| Sele (NM_011345)       | Fw  CAACAAATCCACTGAACAGAAGT  
                        | Rv  CTCCACGGAGGAACAAAA  
                        | Probe  CAGTCTAGCGCCTGGATGAAAGCAAC |
| Selp (NM_011347)       | Fw  CAGAAAGAAAGATGGATGGAAAATG  
                        | Rv  CGGGTTCCTTGGAAGGTTG  
                        | Probe  TTGAACCCCTTCAGCAGCTAGGAAC |
| Tnf (NM_013693)        | Fw  TCTATGCGCCAGACCCCTCAG  
                        | Rv  TTGCTACGACGTGGGCTACA  
                        | Probe  CTCAGATCATCTTTCTCAAAATTCAGTGACAAGC |

Probes were labeled with FAM or Yakima Yellow dye and TAMRA or BHQ1 quencher.
Mouse peritoneal neutrophil isolation and MMP-9 release assay

Mouse neutrophils were obtained as previously described by Lecut and coworkers. ApoE-/- and ApoE-/-FAAH-/- female mice (10-12 weeks of age) were used. The peritoneal lavage fluids from two different mice were pooled for each assay. Mouse neutrophils were resuspended in serum-free RPMI 1640 medium containing 25 mmol/L Hepes. Then, mouse neutrophils (4x10^5 cells per well) were cultured in the presence or absence of 10 ng/ml phorbol myristate acetate (PMA; Sigma), 100 nM fMLP (Sigma), 10 ng/ml TNF-alpha, 10 ng/ml CXCL1, 10 ng/ml CXCL2, or 5 ng/ml CCL3 (all from R&D Systems) for 30 min at 37ºC in a humidified atmosphere 5% CO2 in polystyrene dishes. Levels of pro-MMP-9 in neutrophil supernatants were measured by colorimetric enzyme-linked immunosorbent assay (ELISA, MMP900B, R&D Systems). The limit of detection for pro-MMP-9 was 31.3 pg/ml. Mean intra- and inter-assay coefficients of variation (CV) were below 5%.

Pro-MMP-9 zymographic assay in mouse neutrophil supernatants

Pro-MMP-9 zymographic activity was assessed in mouse neutrophil supernatants as previously described. SDS-polyacrylamide gels (9%) were copolymerized with gelatin (Sigma). Equal amounts of cell culture supernatants (30 µl) and 1 ng of recombinant human pro-MMP-9 standard (Calbiochem) were loaded on gels in the absence of reducing agents. Then, gels were rinsed and stained with Coomassie Blue R-250 as previously described. Gelatinolytic bands were measured with a gel analysis system (GeneGenius, Syngene). Zymographic results were expressed as pro-MMP-9 proteolytic activity and calculated with the following formula: (gelatinolytic activity in supernatants divided by gelatinolytic activity of standard) multiplied by the weight (1 ng) of loaded standard onto the gel.
Migration assay

After isolation, neutrophils from ApoE-/ and ApoE-/FAAH-/- 10-12 week old female mice were resuspended at a density of $1 \times 10^6$ cells/ml in HBSS containing 1 mM CaCl$_2$, 2 mM MgCl$_2$ (ICN Biomed) and 0.2% BSA (Sigma Aldrich). Mouse neutrophil chemotaxis was assessed in a 48-well microchemotaxis chamber using a 5 µm pore size, 5-µm-thick polyvinylpyrrolidone-free polycarbonate filter (Neuro Probe). Neutrophils ($4 \times 10^5$) were seeded in the upper well while control medium or chemoattractants (100 ng/ml TNF-α, 200 ng/ml CXCL1, 200 ng/ml CXCL2, or 100 ng/ml CCL3) were added to the lower wells. After 60 min of incubation at 37°C, the filters were removed from the chambers, washed and stained with Diff-Quick (Baxter). The cells of five random fields were counted and the chemotaxis index was calculated by dividing the number of cells migrated towards chemoattractants through the number of cells migrated to medium alone.

Chemokine measurements in mouse serum (ELISA)

Serum levels of CXCL1, CXCL2, CCL3, pro-MMP-9 and TNF-α after 10 weeks HCD feeding were measured by ELISA (R&D Systems,). The limit of detection for CXCL1 was 15.6 pg/ml, 7.8 pg/ml for CXCL2 and CCL3, 31.3 pg/ml for pro-MMP-9 and 31.3 pg/ml for TNF-α. Mean intra- and inter-assay coefficients of variation (CV) were below 6% for all neutrophil chemoattractants.

Statistical analysis

All results are expressed as mean (±SEM). Differences between $P$ values below 0.05 were considered significant. Two group comparisons were performed with GraphPad
Prism 5.01 software using unpaired two-tailed t test. For multiple group comparison, one-way ANOVA with Bonferroni’s post test was used. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.
Results

Supplemental Table II. FAAH substrate levels in hearts of ApoE-/- and ApoE-/-FAAH-/- mice.

<table>
<thead>
<tr>
<th>Tissue level</th>
<th>ApoE-/-</th>
<th>ApoE-/-FAAH-/-</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>anandamide, pg/mg</td>
<td>1.39 ± 0.11</td>
<td>2.26 ± 0.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PEA, pg/mg</td>
<td>15.13 ± 0.96</td>
<td>28.71 ± 1.11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OEA, pg/mg</td>
<td>19.22 ± 1.34</td>
<td>26.42 ± 1.29</td>
<td>0.0011</td>
</tr>
<tr>
<td>2-AG, pg/mg</td>
<td>263.7 ± 22.35</td>
<td>250.8 ± 9.71</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Tissue levels of FAAH substrates as well as the non-FAAH substrate 2-AG were measured in hearts of 10 week old mice. N=10; n.s., non-significant.

Supplemental Table III. Characteristics of study groups.

<table>
<thead>
<tr>
<th>Weeks of diet</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>ApoE-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22.0 ± 0.4</td>
<td>26.3 ± 1.0</td>
<td>29.5 ± 0.8</td>
<td>28.3 ± 0.4</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>371.1 ± 20.2</td>
<td>751.4 ± 68.3</td>
<td>577.2 ± 50.3</td>
<td>962.1 ± 76.8</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td></td>
<td></td>
<td>189.7 ± 17.04</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>33</td>
<td>10</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>ApoE-/-FAAH-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.8 ± 0.3***</td>
<td>29.5 ± 0.7</td>
<td>30.7 ± 0.4</td>
<td>28.2 ± 0.4</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>218.3 ± 23.2***</td>
<td>762.2 ± 59.7</td>
<td>550.9 ± 41.9</td>
<td>649.8 ± 41.1</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td></td>
<td></td>
<td>183.9 ± 19.33</td>
<td></td>
</tr>
</tbody>
</table>

***P<0.001 for two group comparison between genotypes at 0 weeks.
Supplemental Figure I. Plaque MMP-9 expression correlates with neutrophil infiltrates.

Colocalization in aortic sinus plaques of 10 week HCD-fed ApoE-/− and ApoE-/−FAAH-/− mice was analyzed by fluorescence immunostaining for macrophages (CD68, red) and neutrophils (Ly6G, red) with MMP-9 (green). Nuclei were counterstained with DAPI. White arrows indicate colocalization. The high magnification reveals that MMP-9 staining mostly appears intracellular stored in granules.
Supplemental Figure II. FAAH deficiency does not affect the percentage of Ly6C^{hi} monocytes

Flow cytometric analysis of whole blood and digested aortic arches after 10 weeks HCD (n=7-8) to determine the percentage of Ly6C^{hi}-expressing monocytes. (A-B) Monocytes were identified as CD11b^{hi}CD90^{lo}B220^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo} cells. (B) In addition, monocytes were identified as F4/80^{lo}, I-Ab^{lo} and CD11c^{lo} to exclude macrophages and dendritic cells.
Supplemental Table IV. Systemic levels of neutrophil chemoattractants after 10 weeks diet.

<table>
<thead>
<tr>
<th>Serum markers</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;FAAH&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3, pg/ml</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td></td>
</tr>
<tr>
<td>CXCL1, pg/ml</td>
<td>297.87 ± 35.98</td>
<td>297.28 ± 34.30</td>
<td>0.9906</td>
</tr>
<tr>
<td>CXCL2, pg/ml</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td></td>
</tr>
<tr>
<td>Pro-MMP-9, pg/ml</td>
<td>27.65 ± 3.89</td>
<td>30.72 ± 4.83</td>
<td>0.6219</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM, n=17-19 for ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>FAAH<sup>−/−</sup> (<LOD, inferior to the lower limit of detection).
Supplemental Figure III. Chemotactic migration of murine neutrophils.

Chemotactic response of neutrophils from ApoE-/- (white bars) and ApoE-/-FAAH-/-mice (black bars) to TNF-α, CXCL1, CXCL2 or CCL3.
Supplemental Figure IV. Neutrophil pro-MMP-9 release in response to various stimuli.

Levels of pro-MMP-9 (ng/ml) in cell culture supernatants from ApoE-/- (white bars) and ApoE-/-FAAH-/- neutrophils (black bars) cultured in adherent (polystyrene) condition and stimulated with PMA, fMLP (positive controls) or various mouse chemokines. CTL, medium alone.
Supplemental Figure V. Neutrophil pro-MMP-9 activity as assessed by zymography.

MMP-9 activity in neutrophil culture supernatants were assessed by zymography, and densitometric quantification of both subunits was performed. ST, MMP-9 standard; CTL, unstimulated control.
References


